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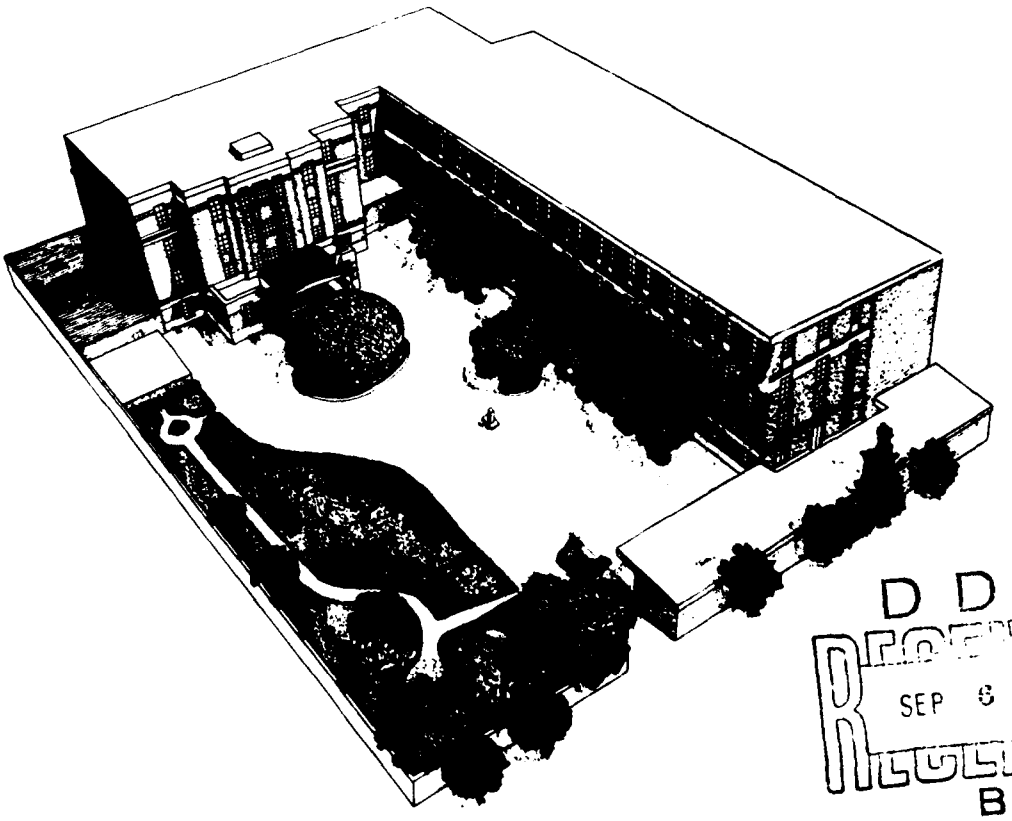

United States Naval
Medical Research Unit No. Two
Taipei, Taiwan

NAMRU-2-TR-503
MARCH 1971

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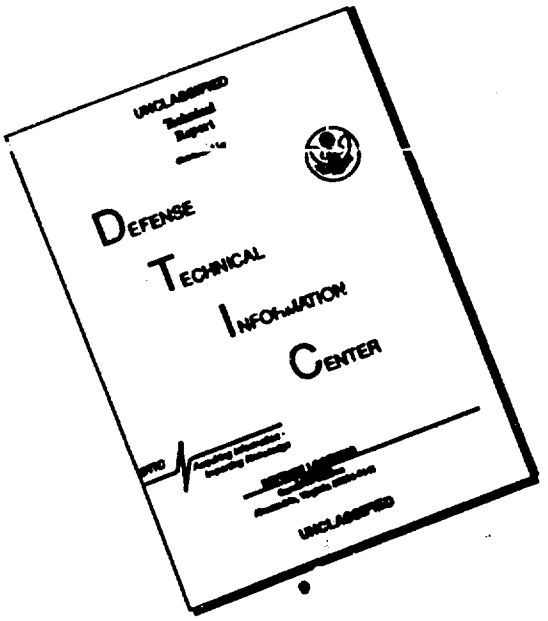

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THIS STUDY WAS SUPPORTED THROUGH FUNDS PROVIDED BY THE BUREAU OF MEDICINE AND SURGERY, NAVY DEPARTMENT, WASHINGTON, D. C.

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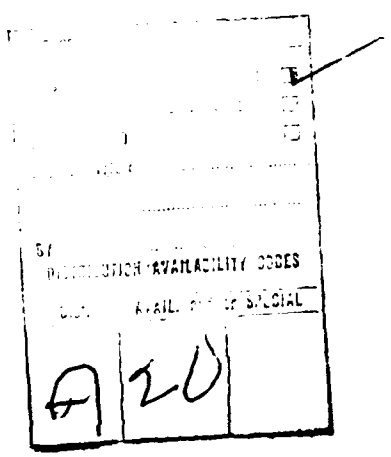

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DOCUMENT CONTROL DATA - R & D

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1 ORIGINATING ACTIVITY (Corporate author)

U. S. Naval Medical Research Unit No. 2
Box 14, APO San Francisco 96263

2a REPORT SECURITY CLASSIFICATION
UNCLASSIFIED
2b GROUP

3 REPORT TITLE

A Prototype Live Oral Cholera Vaccine

4 DESCRIPTIVE NOTES (Type of report and inclusive dates)

Technical Report

5 AUTHOR(S) (First name, middle initial, last name)

Bruce D. Howard

6 REPORT DATE	7a TOTAL NO OF PAGES	7b NO OF REFS
March 1971	3	20

8a CONTRACT OR GRANT NO	9a ORIGINATOR'S REPORT NUMBER(S)
b PROJECT NO	NAMRU-2-TR-503
c	9b OTHER REPORT NO(S) (Any other numbers that may be assigned this report)
d	

10 DISTRIBUTION STATEMENT

Distribution of this document is unlimited

11 SUPPLEMENTARY NOTES	12 SPONSORING MILITARY ACTIVITY
Published in Nature 230(5289): 97-99, Mar. 1971	Bureau of Medicine and Surgery Department of the Navy Washington, D. C. 20390

13 ABSTRACT

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DD FORM 1473 (PAGE 1)
S/N 0101-807-6801

UNCLASSIFIED
Security Classification

KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
V. CHOLERA ORAL VACCINE COPROANTIBODY						

(Reprinted from Nature, Vol. 230, No. 5289, pp. 97–99, March 12, 1971)

A Prototype Live Oral Cholera Vaccine

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Using the methods of molecular genetics it should be possible to develop a cholera vaccine that produces long lasting immunity. To this end *Vibrio cholerae* mutants deficient in choleragenic activity have been isolated.

THE cholera vaccines that are now available have been unsatisfactory for controlling the disease in countries where it is endemic. They do not produce adequate long lasting immunity (more than approximately 3–6 months) with one dose[1,2], and it is impractical to give multiple revaccinations on a massive scale in the affected areas. I describe here procedures for the development of a vaccine with long lasting efficacy.

Cholera victims suffer from a fulminant diarrhoea resulting in severe dehydration and electrolyte imbalance. The diarrhoea is probably caused by a protein factor (toxin) produced by the causative agent, *Vibrio cholerae*[3,4]. This choleragenic toxin can be assayed by its ability to cause in animals a simulated diarrhoea; that is, accumulation of fluid in a ligatured segment of small intestine when the factor is injected into the lumen of the segment. In the canine system the parameters of the fluid and electrolyte alterations are similar to those obtained when live vibrio cells are injected into the intestinal lumen[5,6]. The toxin quickly and apparently irreversibly binds to some substance in the intestinal mucosa. Antibodies directed against the toxin neutralize its activity in the intestinal segment if they are injected before the toxin, but are ineffective if injected 5 min after injection of the toxin[7]. These results parallel the clinical findings in human cholera for which the incubation period can be as short as 1 day[8] and, once the diarrhoea starts, little can be done to shorten substantially its duration or magnitude. (Therapy is basically directed at maintaining fluid and electrolyte balance until the diarrhoea, which is self-limiting, ceases.)

The existing vaccines are preparations of killed vibrio whole cells or cell wall products administered by injection, and probably confer protection by inducing intestinal antibodies (coproantibodies) that act on the bacteria, presumably with a vibriocidal (bacteriocidal) effect; the degree of immunity does correlate well with serum vibriocidal antibody titre[9]. It is thus not surprising that these vaccines produce immunity for only a few months. Unless the antibody titre is already sufficiently high to kill them very quickly, infecting vibrios would produce toxin that would bind to the intestine, causing an outpouring of fluid before a secondary anamnestic response could produce enough antibodies to have a protective effect. An inactivated choleragenic toxin (toxoid) vaccine, which is now being prepared for field testing (personal communication from J. Seal), might somewhat prolong the period of protection but is unlikely to produce long lasting immunity for the same reasons that apply to the existing vaccines.

A vaccine is required which will continue to act antigenically for a prolonged period. This can be accomplished with a particular kind of oral, live bacterial vaccine. The idea of using a live vaccine for cholera, in itself, is not new. Mukerjee and co-workers have been studying naturally occurring non-pathogenic vibrios for this purpose[10]. It should, however, be possible to improve greatly the efficacy of live cholera vaccines by preparing them from bacteria in which non-pathogenicity has been induced by a very specific genetic mutation. The mutation would be in the structural gene for choleragenic toxin and would result in the production of a protein deficient in all biological activity (with respect to diarrhoea production) but with normal antigenic activity. The mutant strain growing in the intestine could induce antibodies directed against the bacteria and the toxin. Immunization could be effected by ingestion of a lyophilized culture of the mutant bacterial strain protected from gastric secretions by an enteric coated capsule, which would remain intact until it reached the small intestine.

Such a vaccine, however, could not provide long term protection because *V. cholerae* do not survive in the intestine for more than a few days. This could be overcome by transferring the mutated structural gene for toxin from *V. cholerae* to a bacterial strain that is normally part of the intestinal flora. The recombinant strain could then permanently reside in the intestine and continually produce the antigen that induces choleragenic toxin neutralizing antibodies. Such inter-species genetic manipulation has been demonstrated for other gram negative bacillary species and genetic recombination between

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different strains of *V. cholerae* has also been reported[11]. The transfer of genetic information for a somatic antigen from *V. cholerae* to *Proteus vulgaris* via a drug resistance factor has been demonstrated (personal communication from L. M. Prescott).

As with any live vaccine, precaution must be taken to ensure that the vaccine strain does not revert to a pathogenic state. The possibility of reversion by back mutation can be eliminated by making the original mutation a "deletion" mutation; that is, one in which a segment of DNA is removed. Deletion mutations do not revert. They can be induced in bacteria by treatment with nitrous acid[12]. Of course the deletion would have to be short enough to retain the antigenicity of the mutant toxin product. Studies of *Escherichia coli* strains carrying mutations in the β-galactosidase gene indicate that a substantial portion of a protein can be missing without its immunological properties being greatly affected[13].

Vibrio deletion mutants could conceivably revert by recombination; that is, by a crossing-over between the DNA of the mutant part of the vibrio toxin gene and some similarly structured portion of a non-homologous gene from another bacterial species that also inhabits the gastrointestinal tract. There is evidence that this occurrence is most unlikely. Reversion has not been observed for other bacterial deletion mutants when extra non-homologous genetic material (e.g., episomes) has been introduced into the mutant cell.

Genetic Studies

Mutants deficient in choleragenic activity were obtained by a method based on the close association of choleragenic activity with another activity, a vascular permeability activity, which also appears in vibrio culture medium filtrates and causes a localized oedema and induration when the filtrate is injected intracutaneously into certain animals[14]. The factor responsible for this second activity has been termed permeability factor (PF). That the association of the two activities continues in varying bacterial growth conditions[15] and separation procedures[3] (only recently has physical separation of the two activities been reported[16]) suggested that some mutants isolated as deficient in PF activity may also have a loss of choleragenic activity. This would occur because either the PF and choleragenic activities, after all, reside on the same protein, or biosynthesis of the two factors is coordinately regulated. In the latter condition, both activities could be eliminated by a single mutation that affects a regulatory gene.

Using this rationale, choleragenic mutants were obtained by nonselectively isolating mutants deficient in PF activity. *V. cholerae* Inaba 569B was mutagenized by three cycles of growth overnight at 37° C in 3% peptone broth containing 60 μg/ml. of N-methyl-N′-nitro-N-nitrosoguanidine (NTG)[17], a potent mutagen. The culture was diluted fifty-fold into fresh medium containing NTG before each start of the next cycle. Although NTG is not known to induce deletion mutants, it was used in this preliminary study instead of nitrous acid because of its much greater potency. Using this method the fraction of cells resistant to 25 μg/ml. of streptomycin increased from $< 10^{-8}$ to 10^{-6}.

The mutagenized cells were cloned and individual colonies were tested for production of PF activity. To minimize isolating mutants that produced a low level of PF factor only because of poor bacterial growth, colonies with irregular morphology and size were avoided. The colonies were inoculated into 1 ml. of peptone broth and incubated at 37° C in a reciprocal shaker. After a culture became noticeably turbid the culture medium was cleared of living bacteria by centrifuging and shaking with a few drops of chloroform. A sample (0.1 ml.) was injected intracutaneously into a rabbit and 16–24 h later the oedema of each injected area was compared with that for a control area, which had been injected with fresh sterile medium also treated with chloroform. Test areas showing no more oedema than the control were scored as negative. The control area usually had no oedema. The colonies exhibiting no activity were again cultured and tested as above. A total of 2,000 colonies were examined in this way. This initial screening procedure was crude and insensitive. For any one rabbit, 10–30% of the injected areas were negative on the first test and of these almost as high a percentage was negative on the repeat test. Nevertheless most strains that were negative twice in the initial screening procedure were also negative by the following more sensitive test. Each strain was inoculated into 10 ml. of peptone broth in a 250 ml. Erlenmeyer flask, which was incubated overnight at 30° C in a rotary incubator. The next day, after being cleared of bacteria by centrifugation and 'Millipore' filtration, the culture medium was assayed for PF using the procedure of Craig[14]. In this procedure the permeability effect is more precisely quantified by intravenous injection of a dye 23 h after the injection of PF. The dye attaches to plasma proteins passing into the oedematous area resulting in a coloured spot on the surface of the skin. In the present study Evans blue dye was used. The unit of PF activity is the blueing dose. One blueing dose is the least amount of PF that will result in a blue spot of 5 mm diameter. Strains that produced less than 5% of the wild type PF activity were tested for choleragenic activity. A 2 ml. sample of a culture, which had been incubated overnight at 30° C in a rotary shaker as I have described, was injected into a 10 cm long ligatured segment of rabbit ileum. After 16–18 h the volume of fluid in the lumen of the segment and the weight of the segment after removal of fluid were determined. Of the forty-three mutant strains that eventually proved to be deficient in PF activity, thirty had the same growth rate as wild type as measured turbidimetrically. Of these, eighteen exhibited no choleragenic activity. The data for four of these mutants are given in Table 1.

Since mutants deficient in PF activity could be obtained so easily, an attempt was made to isolate choleragenic toxin *mutants* directly. The bacteria were grown through three cycles of NTG-containing peptone broth, cloned, and tested for choleragenic activity as we have described. Of the 100 colonies tested, two had detectably decreased choleragenic

Table 1 Production of PF and Choleragenic Activity

Strain	Blueing doses/ml. of culture media filtrate	Fluid accumulation (ml./g of intestine)
Wild-type	1,640	7.6
T30	< 10	< 0.1
T34	< 10	< 0.1
T64	< 10	< 0.1
B56	< 10	< 0.1

Table 2 Induction of Vibriocidal Antibodies by Mutant Strains

Rabbit	Injected strain	Serum vibriocidal antibody titre 1st week	2nd week	3rd week	4th week	5th week
1	T30	10^1	10^1	10^7	10^6	10^7
2	T34	10^1	10^1	10^5	10^5	10^6
3	T64	10^1	10^5	10^5	10^7	10^6
4	B56	10^1	10^6	10^5	10^7	10^6

Two parts log phase culture of wild type 569B, one part serum (each appropriately diluted in normal saline) and one part guinea-pig complement diluted 1 : 20 in normal saline 0.1% peptone solution were incubated for 1 h at 37° C. The final concentration of bacteria was 1,000 to 3,000 cells per ml. After incubation, 0.1 ml. was spread in duplicate on brain heart infusion (Difco) agar plates, incubated overnight at 37° C, and the colonies were counted. The control sample had normal saline substituted for serum. The titres listed are the highest ten-fold serial dilution of serum that permitted a bacterial survival less than 25% of that for the control. Antibody titres listed under the column designated first week are the original preinjection titres.

Table 3 Induction of Vibriocidal Antibodies by Live and Dead Strains

Rabbit	Injected strain	Serum vibriocidal antibody titre			
		1st week	2nd week	3rd week	4th week
5	T34	10^1	10^2	—	10^4
6	T34 (dead)	10^1	10^3	—	10^4
7	T34	10^1	10^3	10^3	10^3
8	T34 (dead)	10^0	10^2	10^3	10^3

activity. One mutant strain caused accumulation of 0.4 ml. of fluid/g of intestine and has a decreased PF production, both of which could be attributed to poor growth in broth. The other had a normal growth rate in broth, produced a normal level of PF activity and only a slightly reduced level of choleragenic activity (fluid accumulation of 1.4 ml./g of intestine). Neither mutant strain would be useful for a vaccine but this study demonstrates that choleragenic toxin mutants can be isolated directly with some ease.

The attempts to transfer the gene for choleragenic toxin from *V. cholerae* to *E. coli* were unsuccessful. *E. coli* was chosen because it is a member of the intestinal flora, its genetics is the best characterized, and it is sufficiently related to *V. cholerae* to allow drug resistance factors to be transferred between the two species[18]. The techniques of Bhaskaran *et al.*[19] were used in the genetic recombination studies. The plan was to select for recombinants involving genes common to both species (for example, genes for metabolism of amino-acids) and then look for choleragenic activity in the *E. coli* recombinants*. However, whereas intra-species (*V. cholerae* × *V. cholerae* and *E. coli* × *E. coli*) recombination occurred at a normal frequency, no inter-species recombination was detected within the limits of sensitivity, which was 10^{-7} to 10^{-9} depending on the strain and genetic marker selection. There was also no detectable transfer of markers from *E. coli* to *V. cholerae* even when high frequency recombination (Hfr) strains of *E. coli* were used as donors. Crosses involving all combinations of donor and recipient strains were attempted, and selection was made for each marker one at a time. Additional experiments along these lines are required.

Immunological Studies

The four mutant strains described in Table 1 were tested for ability to induce antibodies in conditions simulating vaccination by the oral administration of an enteric coated capsule of lyophilized live bacteria. Overnight broth cultures of bacteria were centrifuged, the bacterial pellet was resuspended in 10% skimmed milk, and 0.5 ml. samples of the bacteria-milk suspensions were lyophilized. Later the lyophilized culture was resuspended in 2 ml. of normal saline, assayed for viable bacteria and injected by means of a 26 gauge needle into the ileum of a rabbit after laparotomy. A blood sample was obtained by cardiac puncture before injection of the bacteria. This procedure was repeated at weekly intervals for a further 3 weeks. At the beginning of the fifth week only a blood sample was taken. Each week between 2×10^7 and 2×10^8 viable bacteria were injected. The serum for each week was titred for vibriocidal and PF neutralizing activity. As Table 2, shows, each strain induced high titres of vibriocidal antibody within 2 weeks of the initial injection. However, none of the sera demonstrated any ability to neutralize the PF activity of a wild type culture medium filtrate even when the serum incubated with the filtrate was diluted as little as ten-fold.

These results suggest that the PF trait was not expressed in the intestine. There is evidence that the bacteria injected into the ileum had a very low survival. Cultures from rectal swabs, obtained from the rabbits every few days after injection of bacteria, failed to grow out vibrio. In another experiment the antigenicity of killed bacteria was compared to that of live bacteria. A lyophilized culture of the mutant strain T34 was resuspended in normal saline, centrifuged, again resuspended in normal saline and divided into two equal portions. One sample was immediately injected into the ileum of a rabbit. The other sample was irradiated with ultraviolet light for 2 h—a dose that killed all the bacteria—and then injected into the ileum of another rabbit. For this experiment, bacteria were injected only during the first week. The results, which are summarized in the first two rows of Table 3, show no difference in the ability of the two cultures to induce vibriocidal antibodies. The experiment was repeated with the live culture suspended in 10 ml. of peptone broth to facilitate multiplication in the gut while the dead culture was suspended in 10 ml. of normal saline. The same results were obtained as described in the last two rows of Table 3.

Production of a Vaccine

Except for the transfer of the gene for choleragenic toxin to another strain, there are no barriers to the development of the proposed vaccine. The proper mutant can surely be obtained. Even if genetic transfer of the toxin gene cannot be accomplished, the mutant vibrio strain should in itself be able to induce a high level of immunity for a short while. Mukerjee's group has obtained evidence that it is possible to induce toxin-neutralizing antibodies by intra-intestinal immunization with a live bacterial vaccine[20]. The ease of administration should make adequate frequent revaccination practical.

The exact genetic sites of the mutations in the mutants I have discussed have yet to be determined. It is interesting that the PF activity mutants differ with respect to the presence or absence of choleragenic activity. Acrylamide gel patterns of media filtrate extracts from cultures of these mutants also differ. These studies will be the subject of a future publication.

I thank S. J. Chang, K. W. Hsu, C. S. Kao, S. N. Lin and Y. L. Lin for technical assistance.

Reprint requests should be sent to: Publications Office, NAMRU-2, Box 14, APO San Francisco 96263.

Received October 12; revised November 28, 1970.

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* Strains used were *V. cholerae* V58P$^-$ and V63P$^-$ and *E. coli* AB57F$^-$, AB257Hfr, AB259Hfr, AB311Hfr, AB312Hfr and AB313 Hfr. These strains have mutations in one or more of the genes for synthesis of threonine, leucine, histidine, isoleucine, valine, arginine, proline, methionine and purine, utilization of maltose, and resistance to streptomycin. P and F are the sex factors for *V. cholerae* and *E. coli* respectively.